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(71) Applicant *(for all designated States except US)*: **BIOGEN, INC.** [US/US]; 14 Cambridge Center, Cambridge, MA 02142 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **DE FOUGEROLLES, Antonin, R.** [CA/US]; 119 Lancaster Terrace, Brookline, MA 02446 (US). **KOTELIANSKI, Victor** [US/US]; 4 Charlesgate East, Apt. 405, Boston, MA 02115 (US).

(74) Agents: **HALEY, James, F., Jr.** et al.; c/o Fish & Neave, 1251 Avenue of the Americas, New York, NY 10020 (US).

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(54) Title: ALPHA1BETA1 ANTAGONISTS FOR TREATMENT OF ATHEROSCLEROSIS

(57) Abstract: Methods of treating atherosclerosis by inhibiting the function or expression of VLA-1. Also included are antagonists of VLA-1 that are useful in the methods.

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ALPHA1BETA1 ANTAGONISTS FOR TREATMENT OF ATHEROSCLEROSIS

FIELD OF THE INVENTION

This invention relates to treating atherosclerosis by blocking the function of $\alpha_1\beta_1$ with an antagonist.

BACKGROUND OF THE INVENTION

Integrins are a group of heterodimeric transmembrane proteins that mediate cell-cell and cell-matrix adhesion. Such adhesion is important in many developmental and physiological processes such as cell differentiation, cell proliferation and cell migration, as well as immune and inflammatory processes. Integrins consist of two noncovalently linked polypeptide chains, α and β. In mammals, there are many different α and β subunits, and their various combinations give rise to a great variety of dimers that exert different functions.

Integrin subfamilies were originally defined on the basis of which β subunit was used to form the heterodimers. The β1-containing integrins are also called VLA molecules, referring to as "very late activation" antigens. VLA-1 to VLA-6 refer to β1 subfamily members containing α1 to α6 (i.e., CD49a to CD49f), respectively. For general review, see *Cellular and Molecular Immunology*, eds. Abul K. Abbas et al., W.B. Saunders Company, Philadelphia, PA, 2000.

$\alpha_1\beta_1$ (i.e., VLA-1) is one of the major collagen receptors, and its expression in the adult is largely confined to mesenchymal cells, such as smooth muscle cells, fibroblasts, hepatocytes and the microvascular endothelium. In general,

$\alpha_1\beta_1$ (i.e., VLA-1) is one of the major collagen receptors, and its expression in the adult is largely confined to mesenchymal cells, such as smooth muscle cells, fibroblasts, hepatocytes and the microvascular endothelium. In general, little $\alpha 1\beta 1$ is detectable on peripheral blood lymphocytes and monocytes. However, increased expression of $\alpha_1\beta_1$ has been observed on activated T-lymphocytes in a variety of chronic inflammatory diseases, for example, in the rheumatoid synovium of arthritis patients, lungs of sarcoidosis patients and atherosclerotic plaques. Interventions that inhibit $\alpha_1\beta_1$ expression and/or functions were shown to be protective in mouse models of several inflammatory pathologies, including hypersensitivity, arthritis and Alport's syndrome.

One significant chronic inflammatory disease is atherosclerosis. It is characterized by irregularly distributed lipid deposits in the intima of large and medium-sized arteries and leukocyte adhesion and infiltration throughout the extracellular matrix around diseased sites. Several mouse models for atherosclerosis have been established. One of such models is ApoE -/- mice, in which both copies of the apolipoprotein E gene are knocked out (Plump et al., *Cell* 71:343-53 (1992)).

SUMMARY OF THE INVENTION

This invention is based on the discovery that α1 integrin subunit and ApoE double knockout ("α1-/-/ApoE-/-") mice show (1) a decrease in atherosclerotic plaque extent and inflammation, and (2) induction of a stable plaque phenotype. A stable plaque phenotype decreases the probability that an antherosclerotic plaque will rupture and thrombose.

Accordingly, this invention embraces a method of treating a subject (e.g., a human) having or at risk of having atherosclerosis (e.g., transplant-related atherosclerosis) by administering to the subject a therapeutically effective amount of a composition comprising an antagonist of integrin $\alpha_1\beta_1$. Such antagonists include compounds (e.g., antibodies or peptides) that inhibit the interaction between the integrin and a ligand. Ligands for VLA-1 include, but may not be limited to, collagen such as collagen Types I, IV, VI, XIII, XIV, and X; laminins; other triple helix proteins; and C1q, a complement protein.

Other antagonists include small molecule compounds that modulate integrin signaling pathways to inhibit activity or function of the integrin. A therapeutically effective amount can be indicated by, for example, the alleviation of symptoms of atherosclerotic conditions, such as a reduction of at least 25% (e.g., at least 30%, 40%, 50%, or 60%) in (i) the total and/or individual atherosclerotic plaque areas, (ii) the numbers of T cells and/or macrophages in the plaques, and/or (iii) the content of lipid core in the plaques; or an increase in plaque stability, e.g., as assessed by an increase of at least 25% (e.g., at least 30%, 40%, 50%, or 60%), in (i) the contents of collagen and/or α-smooth muscle cell actin in the plaques, and/or (ii) the degree of chondral metaplasia in the plaques.

An $\alpha_1\beta_1$ antagonist useful in this method can be an antibody, for example, monoclonal antibody AJH10 (ATCC PTA-3580; deposited on August 2, 2001 with the American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209), hAQC2 (ATCC PTA-3275; deposited on April 18, 2001), haAQC2 (ATCC PTA3274; deposited on April 18, 2001), or hsAQC2 (ATCC PTA3356; deposited on May 4, 2001). All of these antibodies were deposited under the Budapest Treaty. An antagonistic antibody may also be, e.g., monoclonal antibody 1B3 (ATCC HB10536) described in U.S. Patents 5,391,481 and 5,788,966.

The antagonist can also be a small molecule, a peptide (e.g., an α1-I domain peptide, which comprises Val-Gln-Arg-Gly-Gly-Arg (SEQ ID NO:1) or a functional variant thereof; or a peptide whose sequence constitutes the $\alpha_1\beta_1$-binding site on collagen, such as the GFOGER (SEQ ID NO:2) sequence on collagen Type I described in WO 01/73444 and Knight et al., *J Biol Chem* 275: 35-40 (2000)), or a peptide mimetic. The antagonist can also be a small organic molecule that blocks integrin interaction with its ligand or modulates integrin signaling to decrease integrin activity or function in or on a cell, such as those described in Weitz-Schmidt et al., *Nat. Med.* 7:687-692 (2001) and WO 00/48626.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Exemplary methods and materials are described below, although methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present invention. All publications and

other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. The materials, methods, and examples are illustrative only and not intended to be limiting. Throughout this specification and claims, the word "comprise," or variations such as "comprises" or "comprising," will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

Other features and advantages of the invention will be apparent from the following detailed description.

BRIEF DESCRIPTION OF THE FIGURES

The asterisks in the following figures indicate that the *p* value of statistical significance is smaller than 0.05.

Fig. 1 is a bar graph showing the total plaque areas (μm^2) in α1-/-/ApoE-/-, α1+/-/ApoE-/-, and α1+/+/ApoE-/- mice.

Fig. 2 is a bar graph showing the individual initial ("ini") and advanced ("adv") plaque areas in α1-/-/ApoE-/-, α1+/-/ApoE-/-, and α1+/+/ApoE-/- mice.

Fig. 3 is a bar graph showing the T cell ($CD3^+$) counts in the initial and advanced atherosclerotic plaques in α1-/-/ApoE-/-, α1+/-/ApoE-/-, and α1+/+/ApoE-/- mice.

Fig. 4 is a bar graph showing the macrophage counts in the initial and advanced atherosclerotic plaques in α1-/-/ApoE-/-, α1+/-/ApoE-/-, and α1+/+/ApoE-/- mice.

Fig. 5 is a bar graph showing the collagen content in the initial and advanced atherosclerotic plaques in α1-/-/ApoE-/-, α1+/-/ApoE-/-, and α1+/+/ApoE-/- mice.

Fig. 6 is a bar graph showing the α-smooth muscle actin content in the initial and advanced atherosclerotic plaques in α1-/-/ApoE-/-, α1+/-/ApoE-/-, and α1+/+/ApoE-/- mice.

Fig. 7 is a bar graph showing the lipid core content in the initial and advanced atherosclerotic plaques in α1-/-/ApoE-/-, α1+/-/ApoE-/-, and α1+/+/ApoE-/- mice.

Fig. 8 is a bar graph showing the extent of chondral metaplasia in the initial and advanced atherosclerotic plaques in α1-/-/ApoE-/-, α1+/-/ApoE-/-, and α1+/+/ApoE-/- mice.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the discovery that $\alpha_1\beta_1$ (i.e., VLA-1) is directly involved in the pathological development of atherosclerosis, and that a reduction in (or even elimination of) VLA-1 activity significantly alleviates (e.g., mitigates, improves, or reduces) atherosclerosis. This invention is also based on the additional discovery that reduction of VLA-1 activity can render atherosclerotic plaques more stable and thereby decrease the chances for thrombosis and conditions related to thrombosis, such as stroke.

Agents useful in reducing VLA-1 activity include antibodies to VLA-1 or part(s) of VLA-1, e.g., to the α1 subunit of VLA-1. Some preferred agents include the anti-VLA-1 antibodies disclosed in U.S. Patent Applications 60/283,794, filed April 14, 2001 and 60/303,689, filed July 6, 2001. The disclosures of these applications are incorporated herein by reference in their entirety. Other agents include small molecules that block the interaction of VLA-1 to its ligand or modulate integrin cell signaling to decrease a cellular activity or biochemical function associated with VLA-1. Agents useful in this invention also include those that can reduce the expression of VLA-1, such as by gene therapy and antisense technology.

An antibody useful in this invention refers to a full antibody, e.g., an antibody comprising two heavy chains and two light chains, or to an antigen-binding fragment of a full antibody such as a Fab fragment, a Fab' fragment, a $F(ab')_2$ fragment or a F(v) fragment. An antibody of this invention can be a murine antibody or a homolog thereof, or a fully human antibody. An antibody of this invention can also be a humanized antibody, a chimeric antibody or a single-chained antibody. An antibody of this invention can be of any isotype and subtype, for example, IgA (e.g., IgA1 and IgA2), IgG (e.g., IgG1, IgG2, IgG3 and IgG4), IgE, IgD, IgM, wherein the light chains of the immunoglobulin may be of types kappa or lambda. Antibodies useful in this invention can be polyclonal or monoclonal antibodies.

Non-Human Antibodies

The monoclonal antibodies useful in this invention can be generated by well known hybridoma technology. To do so, animals (e.g., mice, rats or rabbits) are immunized with purified or crude $\alpha_1\beta_1$ preparations, or any desired antigenic portion thereof (e.g., the α1-I domain fragment); cells transfected with cDNA constructs encoding α_1 and β_1 or antigenic fragments thereof; cells that constitutively express $\alpha_1\beta_1$; and the like. The antigen can be delivered as purified protein, protein expressed on cells, protein fragment or peptide thereof, or as naked DNA or viral vectors encoding the protein, protein fragment, or peptide. Sera of the immunized animals are then tested for the presence of anti-$\alpha_1\beta_1$ antibodies. B cells are isolated from animals that test positive, and hybridomas are made with these B cells.

Antibodies secreted by the hybridomas are screened for their ability to bind specifically to $\alpha_1\beta_1$ and for any other desired features (e.g., capable of disrupting the interaction between $\alpha_1\beta_1$ and its physiological ligands such as collagen).

Hybridoma cells that test positive in the screening assays are cultured in a nutrient medium under conditions that allow the cells to secrete the monoclonal antibodies into the culture medium. The conditioned hybridoma culture supernatant is then collected and antibodies contained in the supernatant are purified. Alternatively, the desired antibody may be produced by injecting the hybridoma cells into the peritoneal cavity of an unimmunized animal (e.g., a mouse). The hybridoma cells proliferate in the peritoneal cavity, secreting the antibody which accumulates as ascites fluid. The antibody may then be harvested by withdrawing the ascites fluid from the peritoneal cavity with a syringe.

The monoclonal antibodies can also be generated by isolating the antibody-coding cDNAs from the desired hybridomas, transfecting mammalian host cells (e.g., CHO or NSO cells) with the cDNAs, culturing the transfected host cells, and recovering the antibody from the culture medium.

Chimeric Antibodies

The monoclonal antibodies useful in this invention can also be generated by engineering a cognate (e.g., murine, rat or rabbit) antibody. For instance, a cognate antibody can be altered by recombinant DNA technology such that

part or all of the hinge and/or constant regions of the heavy and/or light chains are replaced with the corresponding components of an antibody from another species (e.g., human). Generally, the variable domains of the engineered antibody remain identical or substantially so to the variable domains of the cognate antibody. Such an engineered antibody is called a chimeric antibody and is less antigenic than the cognate antibody when administered to an individual of the species from which the hinge and/or constant region is derived (e.g., a human). Methods of making chimeric antibodies are well known in the art. Preferred constant regions include, but are not limited to, those derived from IgG1 and IgG4.

Fully Human Antibodies

The antibodies useful in this invention also include fully human antibodies. They may be prepared using *in vitro*-primed human splenocytes, as described by Boerner et al., J. Immunol. 147:86-95 (1991), or using phage-displayed antibody libraries, as described in, e.g., U.S. Patent 6,300,064.

Some other methods for producing fully human antibodies involve the use of non-human animals that have inactivated endogenous Ig loci and are transgenic for un-rearranged human antibody heavy chain and light chain genes. Such transgenic animals can be immunized with $\alpha_1\beta_1$ or a desired antigenic fragment thereof, and hybridomas are then made from B cells derived therefrom. These methods are described in, e.g., the various GenPharm/Medarex (Palo Alto, CA) publications/patents concerning transgenic mice containing human Ig miniloci (e.g., Lonberg U.S. Patent 5,789,650); the various Abgenix (Fremont, CA) publications/patents with respect to XENOMICE (e.g., Kucherlapati U.S. Patents 6,075,181, 6,150,584 and 6,162,963; Green et al., *Nature Genetics* 7:13-21 (1994); and Mendez et al., 15(2):146-56 (1997)); and the various Kirin (Japan) publications/patents concerning "transomic" mice (e.g., EP 843 961, and Tomizuka et al., *Nature Genetics* 16:133-1443 (1997)).

Humanized Antibodies

The monoclonal antibodies of this invention also include humanized versions of cognate anti-$\alpha_1\beta_1$ antibodies derived from other species. A humanized antibody is an antibody produced by recombinant DNA technology, in which some or all of the amino acids of a human immunoglobulin light or heavy chain that are not

required for antigen binding (e.g., the constant regions and the framework regions of the variable domains) are used to substitute for the corresponding amino acids from the light or heavy chain of the cognate, nonhuman antibody. By way of example, a humanized version of a murine antibody to a given antigen has on both of its heavy and light chains (1) constant regions of a human antibody; (2) framework regions from the variable domains of a human antibody; and (3) CDRs from the murine antibody. When necessary, one or more residues in the human framework regions can be changed to residues at the corresponding positions in the murine antibody so as to preserve the binding affinity of the humanized antibody to the antigen. This change is sometimes called "back mutation." Humanized antibodies generally are less likely to elicit an immune response in humans as compared to chimeric human antibodies because the former contain considerably fewer non-human components.

The methods for making humanized antibodies are described in, e.g., Winter EP 239 400; Jones et al., *Nature* 321:522-525 (1986); Riechmann et al., *Nature* 332:323-327 (1988); Verhoeyen et al., *Science* 239: 1534-1536 (1988); Queen et al., *Proc. Nat. Acad. Sci.* USA 86:10029 (1989); U.S. Patent 6,180,370; and Orlandi et al., *Proc. Natl. Acad. Sci.* USA 86:3833 (1989). Generally, the transplantation of murine (or other non-human) CDRs onto a human antibody is achieved as follows. The cDNAs encoding heavy and light chain variable domains are isolated from a hybridoma. The DNA sequences of the variable domains, including the CDRs, are determined by sequencing. The DNAs encoding the CDRs are transferred to the corresponding regions of a human antibody heavy or light chain variable domain coding sequence by site directed mutagenesis. Then human constant region gene segments of a desired isotype (e.g, γ1 for CH and k for CL) are added. The humanized heavy and light chain genes are co-expressed in mammalian host cells (e.g., CHO or NSO cells) to produce soluble humanized antibody. To facilitate large scale production of antibodies, it is often desirable to produce such humanized antibodies in bioreactors containing the antibody-expressing cells, or to produce transgenic mammals (e.g., goats, cows, or sheep) that express the antibody in milk (see, e.g., U.S. Patent 5,827,690).

At times, direct transfer of CDRs to a human framework leads to a loss of antigen-binding affinity of the resultant antibody. This is because in some cognate

antibodies, certain amino acids within the framework regions interact with the CDRs and thus influence the overall antigen binding affinity of the antibody. In such cases, it would be critical to introduce "back mutations" (*supra*) in the framework regions of the acceptor antibody in order to retain the antigen-binding activity of the cognate antibody.

The general approach of making back mutations is known in the art. For instance, Queen et al. (*supra*), Co et al., *Proc. Nat. Acad. Sci.* USA 88:2869-2873 (1991), and WO 90/07861 (Protein Design Labs Inc.) describe an approach that involves two key steps. First, the human V framework regions are chosen by computer analysis for optimal protein sequence homology to the V region framework of the cognate murine antibody. Then, the tertiary structure of the murine V region is modeled by computer in order to visualize framework amino acid residues that are likely to interact with the murine CDRs, and these murine amino acid residues are then superimposed on the homologous human framework.

Under this two-step approach, there are several criteria for designing humanized antibodies. The first criterion is to use as the human acceptor the framework from a particular human immunoglobulin that is usually homologous to the non-human donor immunoglobulin, or to use a consensus framework from many human antibodies. The second criterion is to use the donor amino acid rather than the acceptor if the human acceptor residue is unusual and the donor residue is typical for human sequences at a specific residue of the framework. The third criterion is to use the donor framework amino acid residue rather than the acceptor at positions immediately adjacent to the CDRs.

One may also use a different approach as described in, e.g., Tempest, *Biotechnology* 9: 266-271 (1991). Under this approach, the V region frameworks derived from NEWM and REI heavy and light chains, respectively, are used for CDR-grafting without radical introduction of mouse residues. An advantage of using this approach is that the three-dimensional structures of NEWM and REI variable regions are known from X-ray crystallography and thus specific interactions between CDRs and V region framework residues can be readily modeled.

Other antigen-binding agents useful in this invention can also be identified using phage-displayed antibody libraries, such as those described in Smith,

Science 228:1315-7 (1985); U.S. Patents 5,565,332, 5,733,743, 6,291,650, and 6,303,313.

Other Moieties

The antibodies useful in this invention may further comprise other moieties to effect the desired functions. For instance, the antibodies may include a toxin moiety (e.g., tetanus toxoid or ricin) or a radionuclide (e.g., ^{111}In or ^{90}Y) for killing of cells targeted by the antibodies (see, e.g., U.S. Patent 6,307,026). The antibodies may comprise a moiety (e.g., biotin, fluorescent moieties, radioactive moieties, histidine tags, etc.) for easy isolation or detection. The antibodies may also comprise a moiety that can prolong their serum half life, for example, a polyethylene glycol (PEG) moiety.

Other Useful Agents

In addition to antibodies, VLA-1 antagonists useful in the methods of this invention also include any non-antibody compounds that inhibit the function of VLA-1, e.g., by blocking the interaction between VLA-1 and its physiological ligands such as collagen, or by modulating VLA-1-dependent cell signaling. Examples of these compounds are small molecule compounds, peptides, and peptide mimetics, such as those described in WO 01/96365; U.S. Patents 6,326,403 and 6,001,961.

These compounds can be identified using, e.g., combinatorial small molecule libraries, combinatorial antibody libraries, rational drug designs, and traditional organic synthesis followed by screening for antagonism using any method known in the art.

In one example, recombinantly expressed VLA-1 or functional fragments thereof can be used to screen libraries of natural, semisynthetic or synthetic compounds. Particularly useful types of libraries include combinatorial small organic molecule libraries, phage display libraries, and combinatorial peptide libraries. Methods of determining whether components of the library bind to a particular polypeptide are well known in the art. In general, the polypeptide target is attached to solid support surface by non-specific or specific binding. Specific binding can be accomplished using an antibody which recognizes the protein that is bound to a solid support, such as a plate or column.

Alternatively, specific binding may be through an epitope tag, such as GST binding to a glutathione-coated solid support, or IgG fusion protein binding to a Protein A solid support.

Alternatively, the recombinantly expressed VLA-1 or parts thereof may be expressed on the surface of phage, such as M13. A library in mobile phase is incubated under conditions to promote specific binding between the target and a compound. Compounds which bind to the target can then be identified. Alternately, the library is attached to a solid support and the polypeptide target is in the mobile phase.

Binding between a compound and the VLA-1 target can be determined by a number of methods. The binding can be identified by such techniques as competitive ELISAs or RIAs, for example, wherein the binding of a compound to a target will prevent an antibody to the target from binding. These methods are well-known in the art. Another method is to use BiaCORE to measure interactions between a target and a compound using methods provided by the manufacturer. A preferred method is automated high throughput screening, see, e.g., Burbaum et al. *Curr Opin Chem Biol.* 1(1):72-8 (1997), and Schullek et al., *Anal Biochem.* 246(1):20-9 (1997).

Once a candidate compound that binds to a target is identified, one then determines whether the compound inhibits the activity of the target. For instance, the candidate compound can be used to screen for its ability to inhibit K562-α1 dependent adhesion to collagen IV. See, e.g., U.S. Application 60/303,689 (*supra*). In another example, the candidate compound is used to compete for binding of an anti-VLA-1 antibody to (1) a VLA-1-expressing cell, or (2) a molecule containing the α1β1 integrin or a fragment thereof, e.g., the α1-I domain.

Another method to identify VLA-1 antagonists is to use the structure of recombinantly expressed VLA-1 for rational drug design. See, e.g., WO 01/73444. The structure of the recombinant protein may be determined using X-ray crystallography or nuclear magnetic resonance (NMR). See, e.g., WO 00/20459, where the crystal structure of the α1-I domain is described. Alternatively, one could use computer modeling to determine the structure of the protein. The structure can then be used in rational drug design to design potential inhibitory compounds of the

target (see, e.g., Clackson, Mattos et al., Hubbard, Cunningham et al., Kubinyi, Kleinberg et al., all herein incorporated by reference).

Diseased Conditions And Animal Models

The methods and agents of this invention can be used to treat a subject (e.g., a human) having or at risk of having atherosclerosis (e.g., transplant-related atherosclerosis) or other $\alpha_1\beta_1$-mediated cardiovascular diseases such as vasculitis, stroke, or restenosis. The treatments of this invention are effective on both human and animal subjects afflicted with these conditions. Animal subjects to which the invention is applicable extend to both domestic animals and livestock, raised either as pets or for commercial purposes. Examples are dogs, cats, cattle, horses, sheep, hogs and goats.

The efficacy of the anti-VLA-1 antibodies or other VLA-1 antagonists of the invention can be tested in various animal models. In addition to the ApoE-/- model described above, mouse models for atherosclerosis include C57BL/6 LDL-R -/- mice, in which both copies of the low density lipid receptor gene are knocked out (Ishibashi et al., *J Clin Invest* 92:883-93 (1993)); apoE Leiden transgenic mice; apoE R142C transgenic mice; and apoB transgenic mice (see, e.g., Breslow, *Science* 272:684-8 (1996)). Indicators of atherosclerotic pathology include total and individual atherosclerotic plaque areas; the numbers of T cells and macrophages in the plaques; the contents of collagen, α-smooth muscle cell actin and lipid core in the plaques; and the degree of chondral metaplasia in the plaques (see below).

The efficacy of the treatments of this invention in patients may be measured by a number of available diagnostic tools, including physical examination, blood tests, ultrasound, magnetic resonance imaging (MRI), cardiogram, angiogram and CT scan.

Pharmaceutical Compositions

The pharmaceutical compositions of this invention comprise one or more VLA-1 antagonists of the present invention, or pharmaceutically acceptable derivatives thereof, optionally with any pharmaceutically acceptable carrier. The term "carrier" as used herein includes known acceptable adjuvants and vehicles.

According to this invention, the pharmaceutical compositions may be in the form of a sterile injectable preparation, for example a sterile injectable aqueous

or oleaginous suspension. This suspension may be formulated according to techniques known in the art using suitable dispersing, wetting, and suspending agents.

The pharmaceutical compositions of this invention may be given orally, intravenously, subcutaneously, intraperitoneally, intramuscularly, intramedullarily, intra-articularly, intra-synovially, intrasternally, intrathecally, intrahepatically, or intracranially as desired, or just locally at sites of atherosclerosis. The pharmaceutical compositions of this invention may also be administered by inhalation through the use of, e.g., a nebulizer, a dry powder inhaler or a metered dose inhaler. Localized administration of an $\alpha_1\beta_1$ antagonist near an atherosclerotic plaque can be accomplished using catheter or stent technology such as described in U.S. Patents 6,025,477; 5,954,706; 5,323,471; 5,102,402 and 5,087,244.

The dosage and dose rate of the antagonists of this invention effective to produce the desired effects will depend on a variety of factors, such as the nature of the disease to be treated, the size of the subject, the goal of the treatment, the specific pharmaceutical composition used, and the judgment of the treating physician. Dosage levels of between about 0.001 and about 100 mg/kg body weight per day, for example between about 0.1 and about 50 mg/kg body weight per day, of the active ingredient compound are useful. For instance, an antibody of the invention will be administered at a dose ranging between about 0.01 mg/kg body weight/day and about 20 mg/kg body weight/day, e.g., ranging between about 0.1 mg/kg body weight/day and about 10 mg/kg body weight/day, and at intervals of every one to fourteen days. In another embodiment, the antagonist is administered at a dose of about 0.3 to 1 mg/kg body weight when administered intraperitoneally. In yet another embodiment, the antagonist is administered at a dose of about 5 to 12.5 mg/kg body weight when administered intravenously. In one embodiment, an antagonist composition is administered in an amount effective to provide a plasma level of antagonist of at least 10 μg/ml.

Practice of the present invention will employ, unless indicated otherwise, conventional techniques of cell biology, cell culture, molecular biology, microbiology, recombinant DNA, protein chemistry, and immunology, which are within the skill of the art. Such techniques are described in the literature. See, for example, *Molecular Cloning: A Laboratory Manual*, 2nd edition (Sambrook et al.,

Eds.), 1989; *Oligonucleotide Synthesis*, (M.J. Gait, Ed.), 1984; U.S. Patent 4,683,195 to Mullis et al.; *Nucleic Acid Hybridization*, (B.D. Hames and S.J. Higgins), 1984; *Transcription and Translation*, (B.D. Hames and S.J. Higgins), 1984; *Culture of Animal Cells* (R.I. Freshney, Ed.), 1987; *Immobilized Cells and Enzymes*, IRL Press, 1986; *A Practical Guide to Molecular Cloning* (B. Perbal), 1984; *Methods in Enzymology*, Volumes 154 and 155 (Wu et al., Eds.), Academic Press, New York; *Gene Transfer Vectors for Mammalian Cells* (J.H. Miller and M.P. Calos, Eds.), 1987; *Immunochemical Methods in Cell and Molecular Biology* (Mayer and Walker, Eds.), 1987; *Handbook of Experiment Immunology*, Volumes I-IV (D.M. Weir and C.C. Blackwell, Eds.), 1986; *Manipulating the Mouse Embryo*, 1986.

The following example is meant to illustrate the methods and materials of the present invention. Suitable modifications and adaptations of the described conditions and parameters normally encountered in the art and obvious to those skilled in the art are within the spirit and scope of the present invention.

Experimental Data

I. MICE

α1 mice (α1-/- or α1+/-) were produced on a C57BL/6 background using the methods described in Gardner et al., *Dev. Biol.* 175:301-313 (1996). These mice were crossed with ApoE-/- C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME). After multiple backcrosses, the following littermate mice were obtained: α1-/-/ApoE-/-, α1+/-/ApoE-/-, and α1+/+/ApoE-/-.

The animals were fed a normal chow diet for 26 wks. After the experimental procedure, they were sacrificed after a 24 hr fast. Blood (0.5-1 ml) was obtained from the caval vein of the animals for lipoprotein analysis. The arterial tree was perfused, through a catheter inserted into the left ventricular apex, with phosphate buffered saline (PBS) for 3 min and with 1% phosphate buffered paraformaldehyde for 3 min. Both solutions contained 0.1 mg/ml sodium nitroprusside (Sigma).

Then, the aortic arch, including its main branch points (brachiocephalic trunk, left common aortid artery and left subclavian artery) was excised and fixed in 1% phosphate buffered paraformaldehyde. All vessels were processed and embedded longitudinally in paraffin. The entire aortic arch was cut into approximately 40 sections. A series of 20 sections, each 4μm in thickness and representing the central

area of the arch with an intact morphology of the complete arch, was used for analysis.

In addition, more than 10 organs of each mouse were excised, embedded in paraffin, and analyzed on 4 μm H&E-stained sections. The organs that were analyzed included the heart, lungs, liver, gall bladder, spleen, jejunum, ileum, colon, lymph nodes, stomach, esophagus, duodenum, pancreas, salivary gland, testis, bladder and bone.

II. LIPID PROFILE

For the assessment of lipid profiles, standard enzymatic techniques were used and automated on the COBAS FARA centrifugal analyzer (Hoffmann-La Roche, LTD, Basel, Switzerland). The levels of total plasma cholesterol, high density lipid ("HDL"), total glycerol, and free glycerol were measured using Kit Nos. 0736635 (Roche), 543004 (Roche), 337-40A/337-10B (Sigma) and 0148270 (Roche), respectively. PRECIPATH (standardized serum) was used as an internal standard. Low density lipid ("LDL") was calculated as:

Total cholesterol – {[(Total glycerol-Free glycerol)/2.2]-HDL}

III. HISTOLOGY AND MORPHOMETRY

For histological analyses, four sections of the tissue of interest (each 20μm apart) were stained with hematoxylin and eosin. Atherosclerotic lesions were then analyzed and classified according to the American Heart Association (AHA) criteria. Atherosclerotic lesions were then analyzed and classified on the basis of histological criteria as either initial (AHA type II/III; fatty streaks, containing macrophage derived foam cells) or advanced lesions (AHA type IV/V; containing extracellular lipid, a lipid core and/or a fibrous cap). The number of atherosclerotic plaques, the presence or absence of lipid cores and calcification, and the total number of cells per lesion were also determined. Lawson staining (modified Elastica von Giesson, Verhoef staining), which exclusively stains the elastic laminae, was used to delineate the internal elastic lamina, permitting an easier distinction between media and intima.

Four Lawson-stained sections (20 μm apart) were used for morphometric analysis. All morphometric parameters were determined using a microscope coupled to a computerized morphometry system (QUANTIMET 570,

Leica, the Netherlands). The aortic arch region was defined as the aortic root to approximately 1.5 mm caudal from the left subclavian artery branch point. Main branch points were also included into the measurements: the brachiocephalic trunk, left common carotid artery and left subclavian artery. The cut-off points were 1.5 mm cranial to the right subclavian artery branch point, and at the same height for the left common carotid artery. Plaque area, excluding the underlying media, was determined for each individual plaque. The relative lipid core content was determined by dividing lipid core area by plaque area. The number of cells per mm^2 lesion area was also determined. The relative collagen area (that is, the percentage of area that stained positive for Sirius Red) was determined for each atherosclerotic plaque on 2 sections per aortic arch, using the same morphometry system.

IV. IMMUNOHISTOCHEMISTRY

Paraffin tissue sections were labelled with an anti-α-smooth muscle actin mAb ($ASMA^{FITC}$, 1:500; Sigma), where the actin was used as a marker for vascular smooth muscle cells and fibroblasts. An anti-Mac-3 mAb (M3/84, 1:30; Pharmingen) was used to detect macrophages. Anti-CD3 polyclonal antibody (A0452, 1:200 dilution; Dako, Carpenteria, California) was used to detect T-lymphocytes. Factor VIII (1:500; Dako) was used to detect endothelial cells. Double immunohistochemistry was performed with anti-α1 (alexa-488 labelled; Biogen) and cell type specific antibodies (CD11b (Pharmingen), ASMA, CD31 and CD3) to determine α1 expression in normal mouse atherosclerotic plaques.

To determine the relative amounts of T-lymphocytes and macrophages in an atherosclerotic plaque, the numbers of CD3- and Mac-3-positive cells in the entire lesion were divided by the total number of cells in the entire lesion. The relative area of the atherosclerotic plaque positive for α-smooth-muscle actin was determined by dividing the ASMA-positive area by total plaque area, using the morphometry system. All measurements were made by one investigator; intraobserver variation was less than 10%.

V. STATISTICS

Data are expressed as mean±SEM. α1-/-/ApoE-/- mice were compared with α1+/-/ApoE-/- and α1+/+/ApoE-/- mice, and α1+/+/ApoE-/- were compared

with α1-/-/ApoE-/- by a non-parametric Mann-Whitney U-test. Data were considered statistically significant at $p<0.05$.

Results

VI. MOUSE STUDY: GENERAL

Body weight and heart weight did not differ among the three experimental groups. No difference in the levels of plasma cholesterol (11.3±0.9 vs. 10.0±0.7), triglyceride (1858±198 vs. 1736±102), LDL (10.1±0.8 vs. 8.8±0.6) and HDL (0.40±0.06 vs. 0.43±0.08) were seen between α1+/+/ApoE-/- and α1-/-/ApoE-/- mice (α1+/+/ApoE-/- vs. α1-/-/ApoE-/-). The corresponding levels in the α1+/-/ApoE-/- mice were each higher than in the other two groups: plasma cholesterol, 15.8±1.4; triglyceride. 2884±473; LDL, 13.6±1.1; HDL, 0.84±0.09. Autopsy of more than 10 organs (i.e., heart, lungs, liver, gall bladder, spleen, jejunum, ileum, colon, lymph nodes, stomach, esophagus, duodenum, pancreas, salivary gland, testis, bladder and bone) revealed no abnormalities among the three groups.

VII. EXPRESSION OF ALPHA-1

In atherosclerotic plaques found in α1+/+ ApoE-/- mice, the majority of smooth muscle cells stained positive for α1. But a small percentage of T-cells were also positive for α1, whereas in macrophage foam cells and endothelial cells, α1 expression was not detected.

VIII. PLAQUE EXTENT

We analyzed 47 lesions in the aortic arches of the α1-/-/ApoE-/- mice (n=10; "n" for number of mice), 43 lesions in the α1+/-/ApoE-/- mice (n=9), and 66 lesions in the α1+/+/ApoE-/- mice (n=12). In α1-deficient mice, the total plaque area was reduced by 34.1% relative to control mice (α1-/-/ApoE-/-: 217,075±42,062 μm^2; α1+/+/ApoE-/-: 329,448±29,589μm^2; $p<0.05$) (Fig. 1). The mean number of plaques per aortic arch did not change. The decrease in plaque area was mostly due to a 37.7% decrease in advanced atherosclerotic plaque area (α1-/-/ApoE-/-: 79,771±14,039 μm^2; α1+/+/ApoE-/-: 127,999±20,270 μm^2; $p<0.05$) (Fig. 2).

Notably, the absence of only one α1 allele sufficed to reduce the total plaque area significantly. In the α1+/-/ApoE-/- mice, the total plaque area was

reduced by 37.2%, while advanced plaque area was reduced by 24.7% compared to the α1+/+/ApoE-/- mice ($p<0.05$) (Figs. 1 and 2).

IX. PLAQUE COMPOSITION

Besides the decrease in plaque area, the composition of the plaques significantly differed between the α1-/-/ApoE-/- mice and the α1+/+/ApoE-/- or α1+/-/ApoE-/- mice.

A. Initial atherosclerotic plaques

Analysis of initial atherosclerotic lesion development in the aortic arch revealed that the relative numbers of $CD3^+$ cells (T-lymphocytes) (α1-/-/ApoE-/-: 0.8±0.6%; α1+/+/ApoE-/-: 1.1±0.4%; Fig. 3) and macrophages (α1-/-/ApoE-/-: 74.8±3.1%; α1+/+/ApoE-/-: 68.2±5.2%; Fig. 4), as well as the contents of collagen (α1-/-/ApoE-/-: 1.9±0.8%; α1+/+/ApoE-/-: 2.3±0.9%; Fig. 5) and α-smooth muscle actin (α1-/-/ApoE-/-: 0.8±0.6%; α1+/+/ApoE-/-: 1.3±0.6%; Fig. 6), did not change significantly in the α1/ApoE double knockout mice in comparison from ApoE-/- mice. In α1+/-/ApoE-/- mice, the plaque phenotype was also not changed (Figs. 3-6).

B. Advanced atherosclerotic plaques

In advanced lesions, the absence of α1 not only inhibited plaque enlargement, but also altered plaque phenotype as viewed both by H&E staining and SR staining. First of all, the numbers of inflammatory cells in the advanced plaques of the α1/ApoE double knockout mice were significantly decreased as compared to the ApoE-/- mice. The percentage of $CD3^+$ cells in the α1-/-/ApoE-/- mice was 0.3±0.1, whereas the corresponding percentage in the α1+/+/ApoE-/- mice was 2.9±0.3% (Fig. 3). The percentage of macrophages in the α1-/-/ApoE-/- mice was 31.5±4.2%, whereas the corresponding percentage in the α1+/+/ApoE-/- mice was 47.8±3.0% (Fig. 4).

Furthermore, the lipid cores of the α1-/-/ApoE-/- plaques were very small (α1-/-/ApoE-/-: 10.2±1.8%; α1+/+/ApoE-/-: 30.7±1.8%; $p<0.05$) (Fig. 7). The lipid cores seemed to have been replaced by chondral metaplasia and extracellular matrix. Fig. 8 shows that the chondral metaplasia content in the α1-/-/ApoE-/- mice was 19.8±4.4%, compared to 8.1±2.5% in the α1+/+/ApoE-/- mice ($p<0.05$; Fig. 8).

Fig. 5 show that the content of collagen, a major component of the extracellular matrix, increased from 22.9±8.4% in the α1+/+/ApoE-/- mice to 35.8±5.4% in the α1-/-/ApoE-/- mice ($p<0.05$). Fig. 6 shows that the content of α-smooth muscle cell actin, another major component of the extracellular matrix, went from 1.4±0.3% in the α1+/+/ApoE-/- mice to 2.9±0.7% in the α1-/-/ApoE-/- mice ($p<0.05$).

Plaques of the α1+/-/ApoE-/- mice showed an intermediate plaque phenotype. These plaques displayed a significant decrease in $CD3^+$ cells, a trend in decreasing the macrophage number ($p=0.07$), and increases in chondral metaplasia ($p=0.07$) and collagen content ($p=0.06$) (Figs. 3-8).

X. CONCLUSION

We have demonstrated that the absence of α1 not only reduces atherosclerosis extent, but also decreases inflammation. The absence of α1 also results in the development of a more stable plaque phenotype, which decreases the probability of plaque rupture and thrombosis.

What is claimed is:

1. A method of treating a subject having or at risk of having atherosclerosis, comprising administering to the subject a therapeutically effective amount of a composition comprising an antagonist of $\alpha_1\beta_1$.

2. The method of claim 1, wherein the antagonist inhibits the interaction between $\alpha_1\beta_1$ and collagen.

3. The method of claim 1, wherein the antagonist is an antibody.

4. The method of claim 3, wherein the antibody is AJH10 (ATCC PTA-3580).

5. The method of claim 3, wherein the antibody is hAQC2 (ATCC PTA-3275).

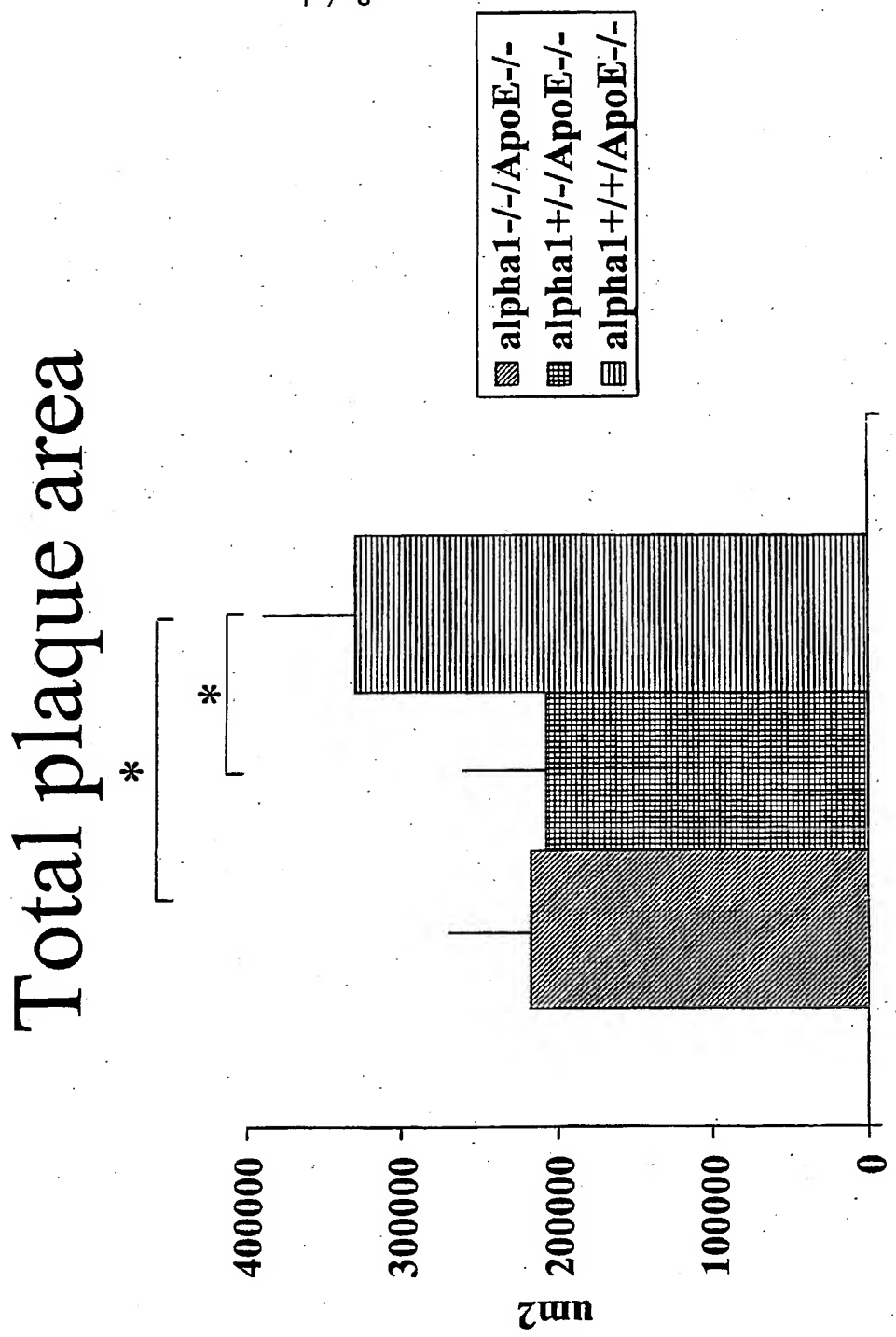
6. The method of claim 3, wherein the antibody is haAQC2 (ATCC PTA3274).

7. The method of claim 3, wherein the antibody is hsAQC2 (ATCC PTA3356).

8. The method of claim 3, wherein the antibody is 1B3 (ATCC HB10536).

9. The method of claim 3, wherein the antibody is a humanized antibody.

10. The method of claim 1, wherein the subject is a human.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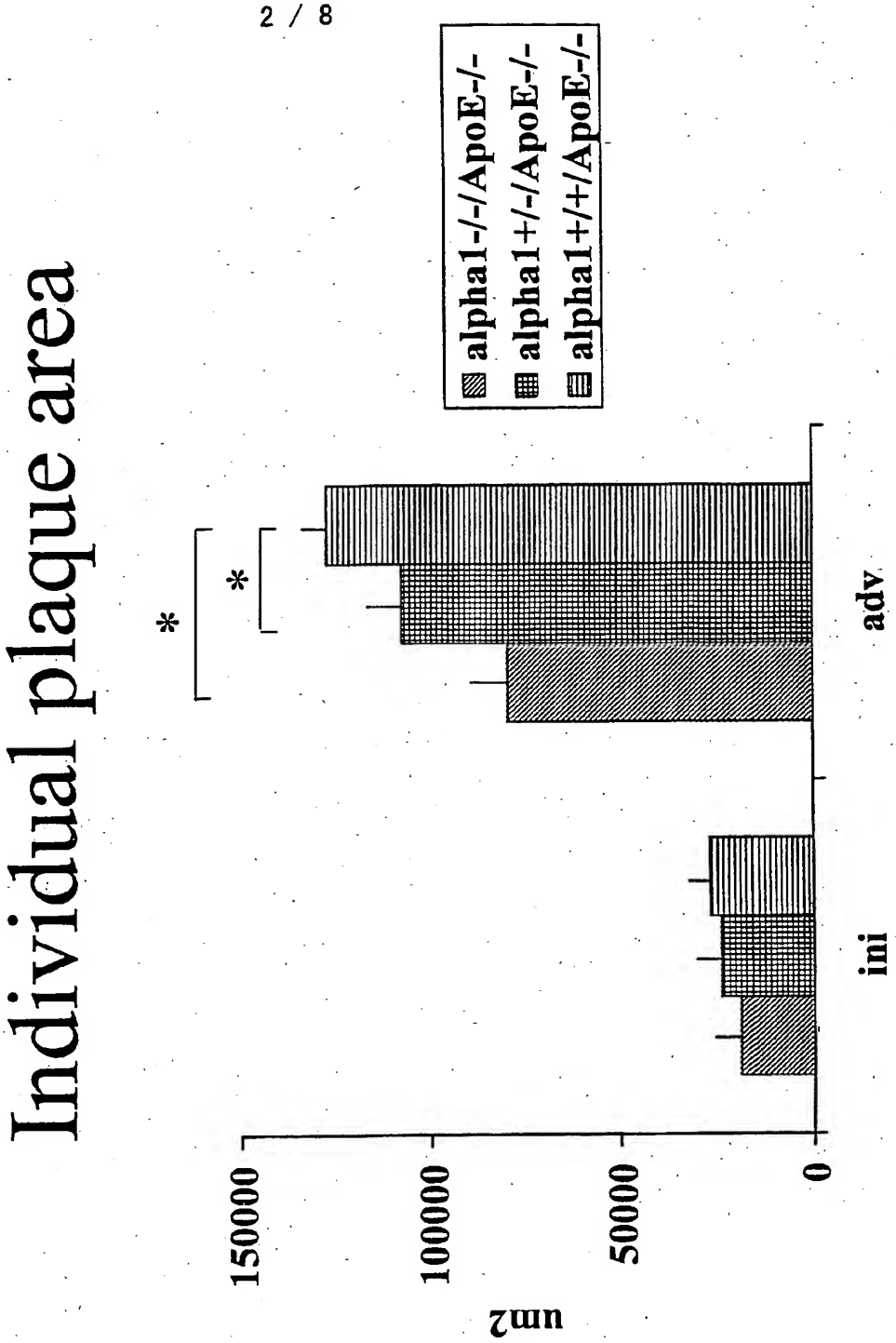
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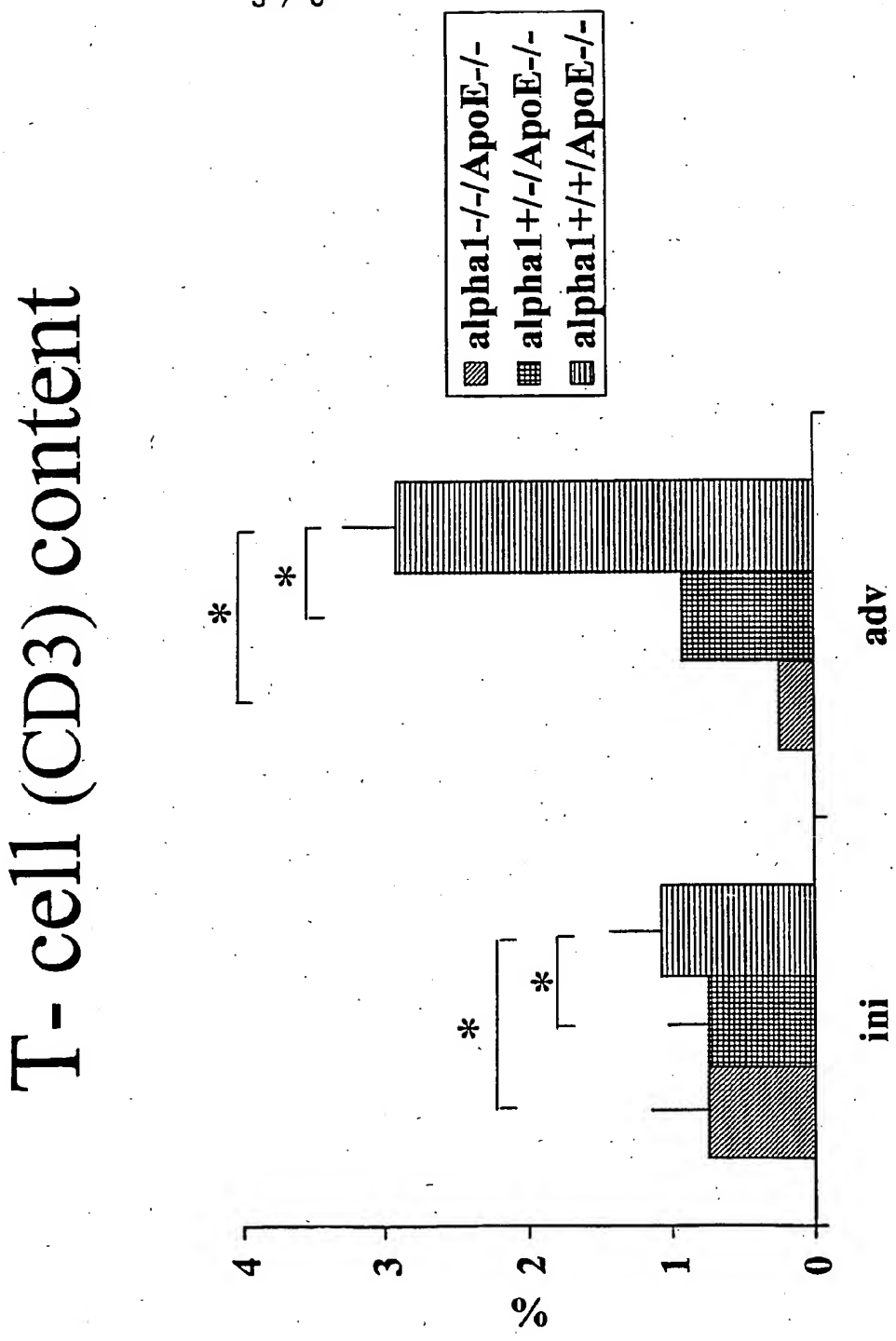
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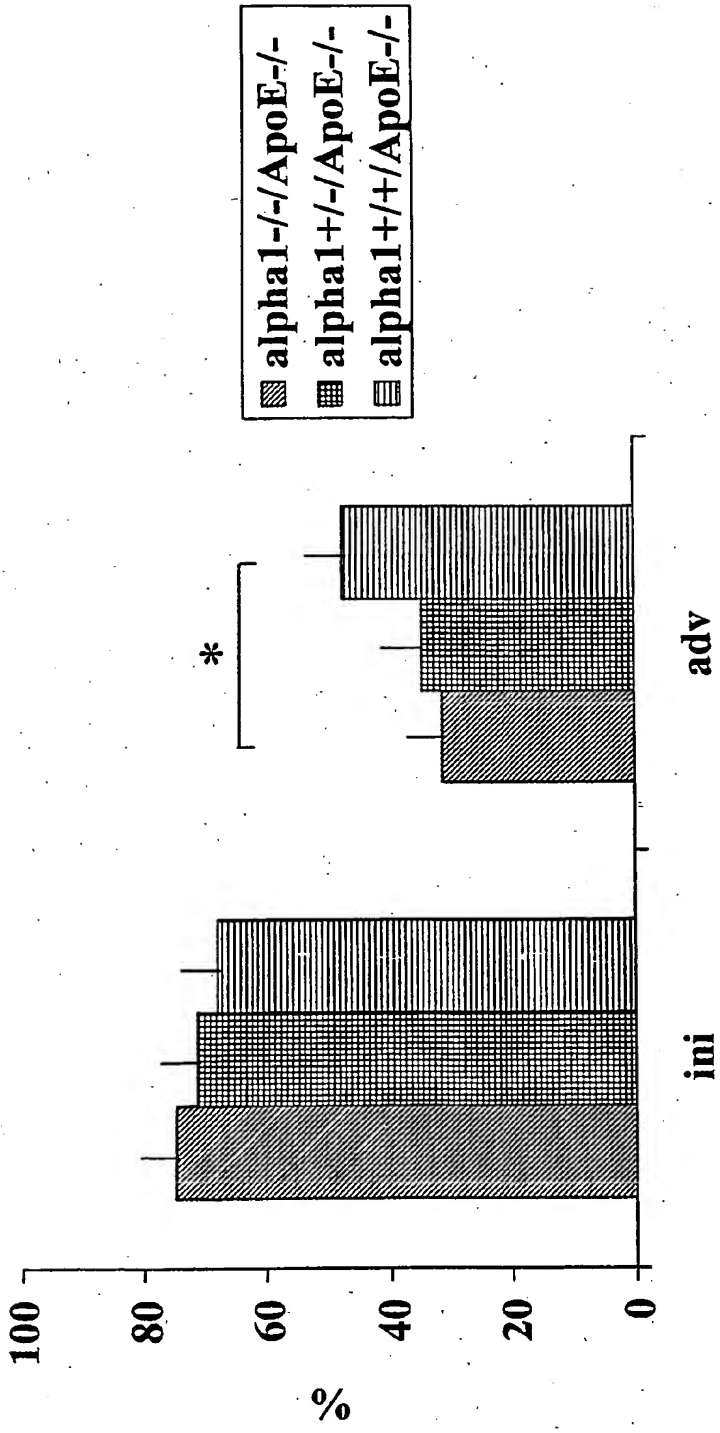

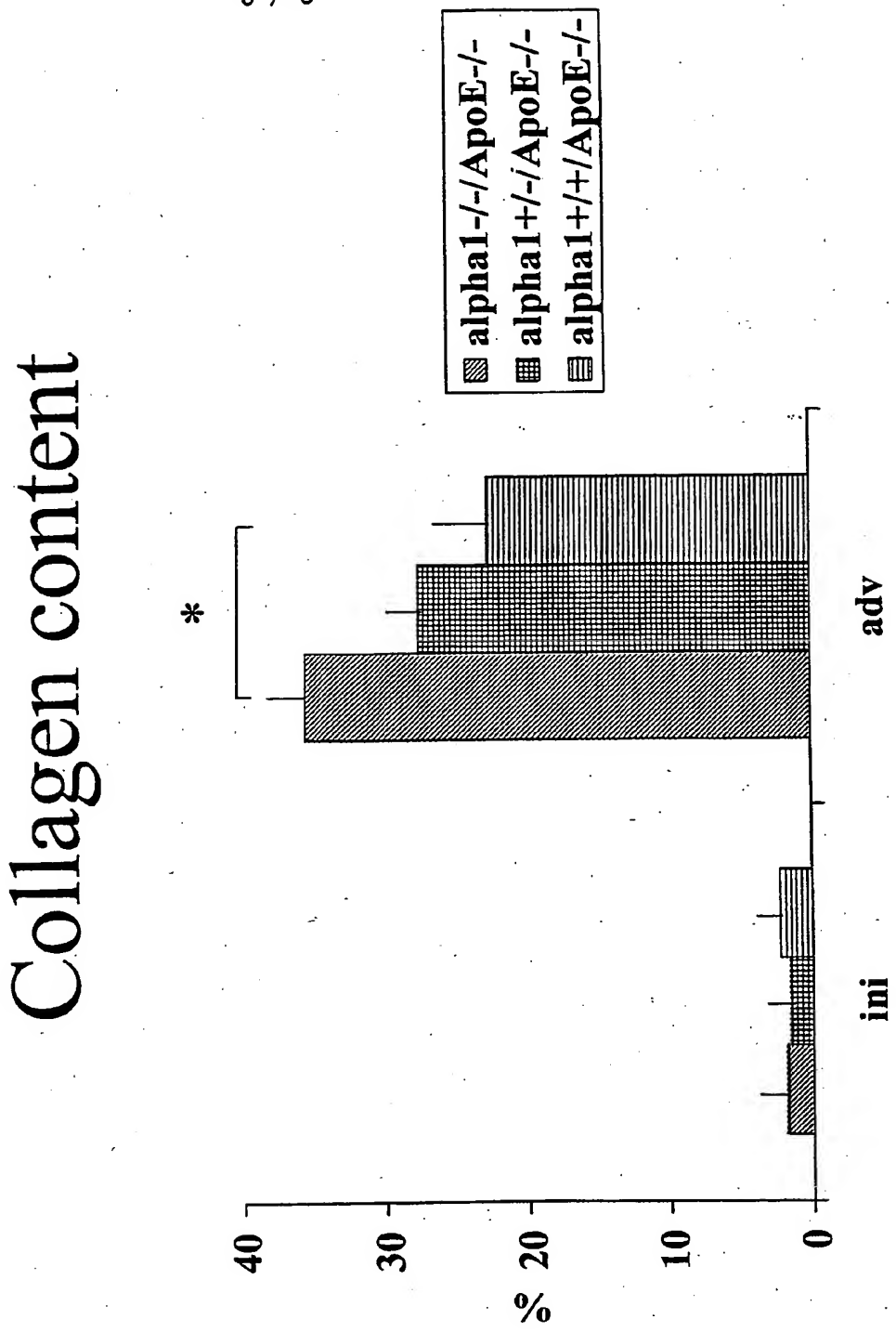
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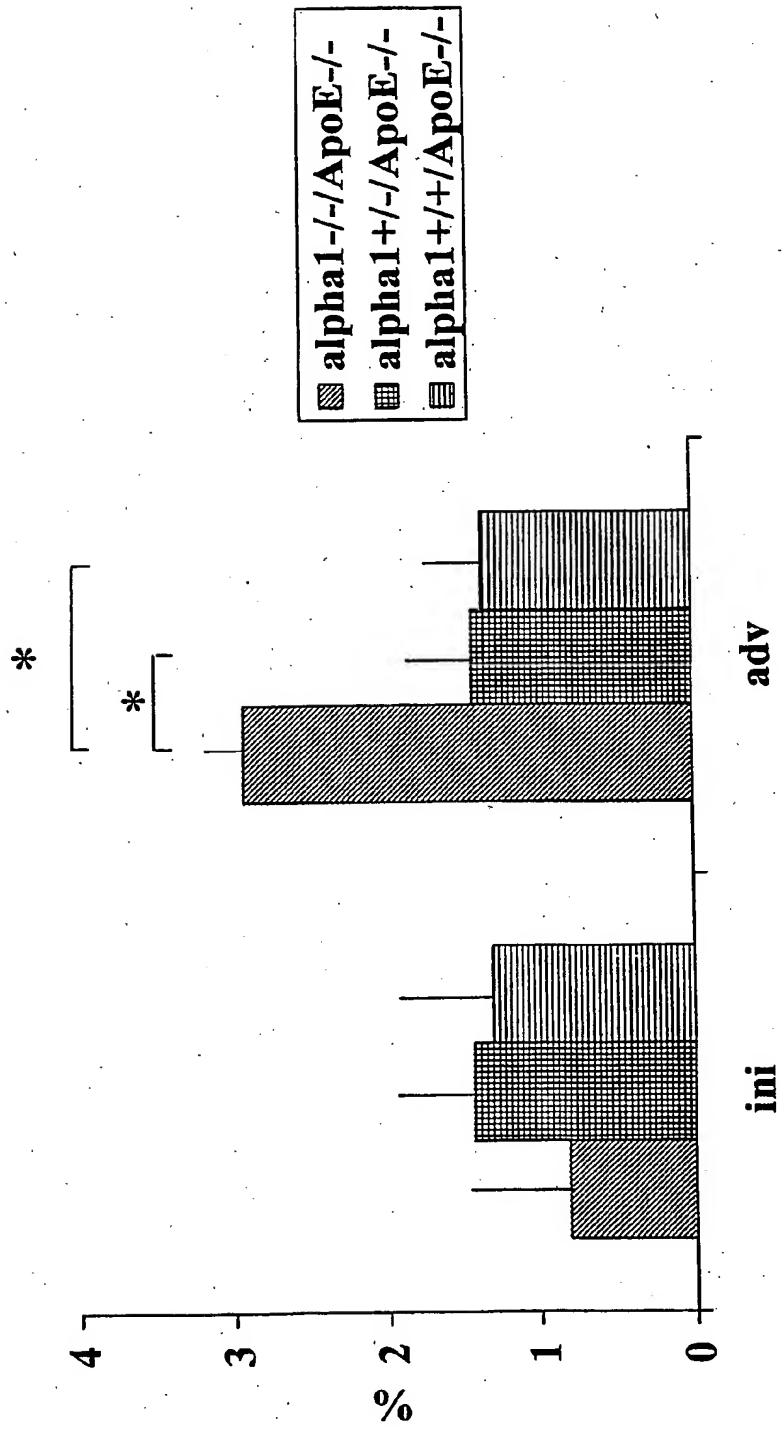
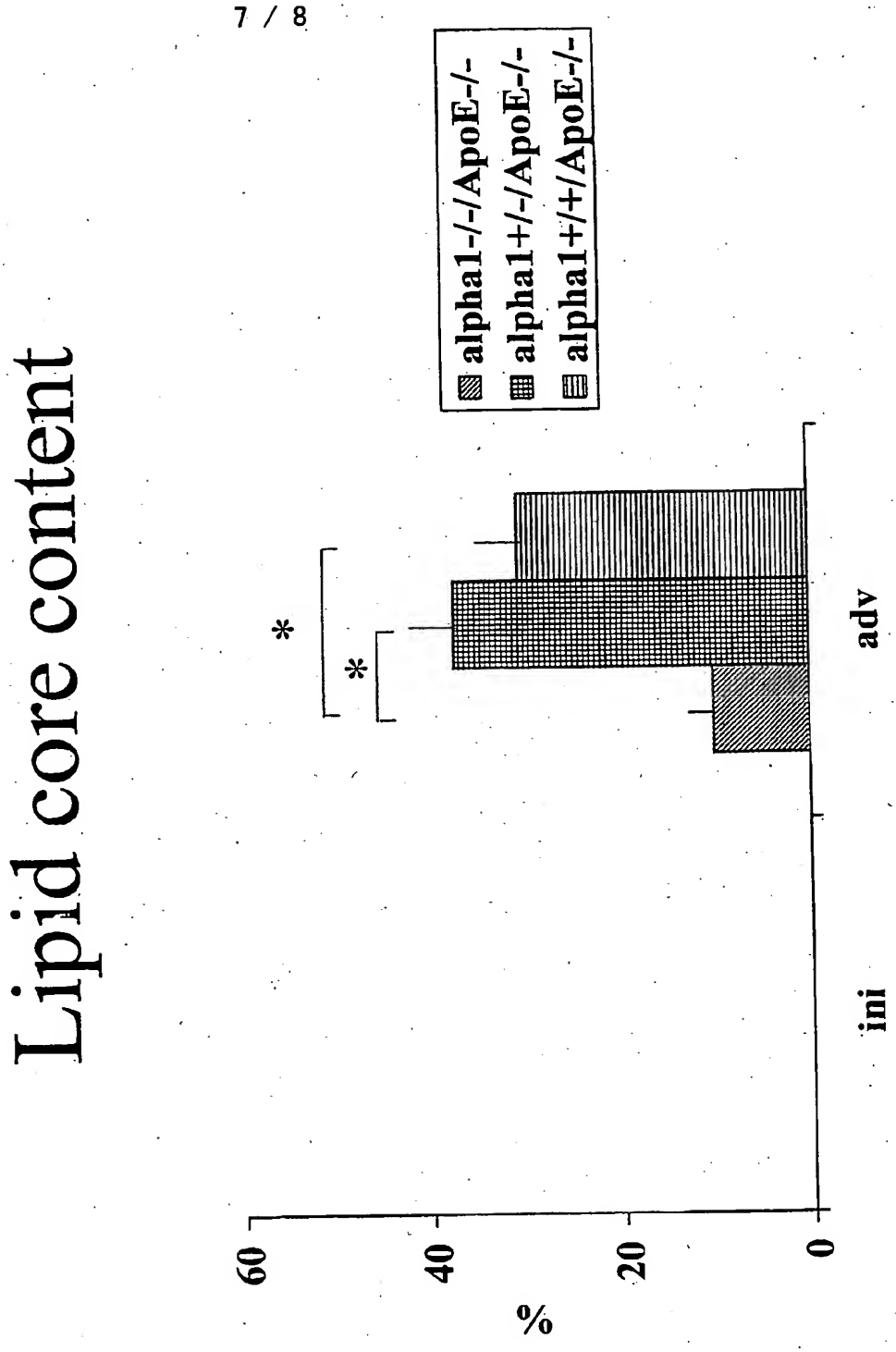
α-smooth muscle actin content

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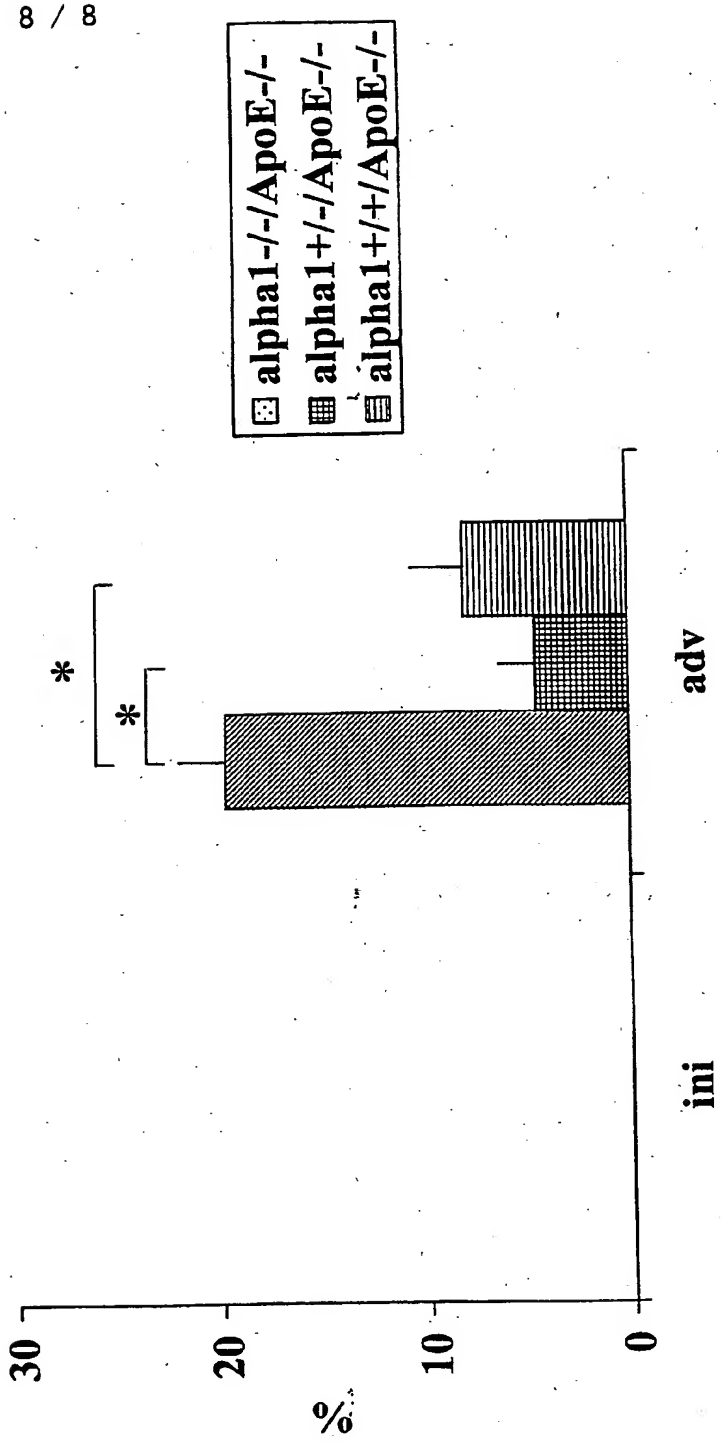
Chondral metaplasia



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US03/04927

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 39/395
US CL : 424/143.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 424/143.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	US 2002/0146417 A1 (GOTWALS et al.) 10 October 2002, (10.10.2002) see entire document, especially column 4, paragraph 0049.	1-4
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Y,P		5-10
Y	MacDonald et al. Journal of Clinical Pathology, 1990, Volume 43, pages 313-315, see entire document.	1-2
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A		3-10

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 June 2003 (02.06.2003)	17 JUN 2003
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (703)305-3230	Authorized officer Donna Jagoe Telephone No. (703) 308-1235

Form PCT/ISA/210 (second sheet) (July 1998)

Continuation of B. FIELDS SEARCHED Item 3:
STN file reg, file medicine, WEST 2.1, search terms, VLA-1, atherosclerosis, arteriosclerosis, alpha-1 beta-1, antagonist near alpha-1, inhibit near alpha-1, collagen, AJH10, hAQC2, haAQC2, hsAQC2, 1B3.